Synthesis and hybridization analysis of a small library of peptide–oligonucleotide conjugates

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ABSTRACT

A small library of 49 peptide–oligonucleotide conjugates were synthesized to explore the influence of various peptide side chains on the hybridization properties of the DNA. An invariant 8mer oligonucleotide was coupled to a peptide portion that contained a five residue variable region composed of the cationic amino acids lysine, ornithine, histidine and arginine, the hydrophobic amino acid tryptophan, and alanine as a spacer. Melting temperature analysis indicated that $T_m$ depended principally on the number of cationic residues. The free energies of binding for polycationic polyarginine peptide giving the most favourable $\Delta G_{vH}$ was found to depend on the presence of positive charge and also the exact identity of the cationic amino acid, with the polyarginine peptide giving the most favourable $\Delta G_{vH}$ value and the most exothermic $\Delta H_{vH}$. Further exploration suggested that the cationic peptide fragments interacted mainly with single-stranded rather than duplex DNA. A study of pH dependence showed that the polyhistidine conjugate was particularly sensitive to pH changes near neutrality, as indicated by a significant rise in $T_m$ from 19.5°C at pH 8.0 to 28.5°C at pH 6.0.

INTRODUCTION

Synthetic oligonucleotides offer the potential for specific control of cellular or viral gene expression at the transcriptional or translational level (1,2). In the search for ‘therapeutic’ oligonucleotides that would meet the requirements needed for medical applications, chemically modified analogues have attracted great interest (3,4). One form of modification that has been explored is the coupling of oligonucleotides to peptide moieties to improve the characteristics of the oligonucleotide. Hybrid molecules composed of peptides and nucleic acids have found use in several applications, such as non-radioactive labels (5), as PCR primers (6) and in encoded combinatorial chemistry (7). Furthermore, they may become important in the field of therapeutic oligonucleotides. Thus, peptides predicted to have favourable interactions with cell membranes, such as polylysine (8), other highly basic peptides (9), hydrophobic peptides (10), viral fusion peptides (11) and peptide signal sequences (12), have been coupled to oligonucleotides in order to enhance their cellular uptake. Peptides able to chelate metals have been appended onto oligonucleotides to generate specific nucleic acid cleavage agents (13). Peptides linked to the 3′-end of oligonucleotides have been reported to provide improved resistance to 3′-exonucleases (10).

An important parameter that helps determine the efficacy of a therapeutic oligonucleotide is its binding affinity for its target nucleic acid strand. Peptide–oligonucleotides have been proposed as dual-specific binding agents resulting in improved target binding affinity and specificity over simple oligonucleotides. Tung et al. prepared antisense peptide–oligonucleotide conjugates between short oligonucleotides (4–6mers) and an arginine-rich peptide sequence derived from HIV Tat peptide (14). This construct was shown to bind specifically to the TAR RNA of HIV-1 in a cell-free system, whereas the unconjugated oligonucleotides were far less effective. The use of shorter therapeutic oligonucleotides would allow for less expensive synthesis, enhance primary sequence mismatch specificity and possibly improve cellular uptake (15).

Proteins that bind to DNA and RNA generally possess some subset of the positively charged amino acids lysine, arginine and histidine within their nucleic acid binding sites. For instance, some histones (e.g. H1) are very lysine rich, whereas others are slightly arginine rich (e.g. H3 and H4) (16,17). Other examples include nucleases (18) and antiterminators (19). Hence the possibility that attachment of cationic peptide sequences to oligonucleotides may improve their target binding properties has been explored by several groups. Corey and co-workers have appended a series of lysine-rich peptides onto oligonucleotides and observed dramatic increases in the rate of association with target duplex DNA (20,21).

Short strings of arginine and ornithine have been linked to oligonucleotides and observed to increase $T_m$ values when bound to target strands (22,23). Stabilization of triplex formation has also been reported for tetrapeptide–oligonucleotide hybrids where the peptide portions possess four positive charges at physiological pH (24). Wei and colleagues have demonstrated the synthesis of oligonucleotide–polyarginine–oligonucleotide constructs (25). The cationic peptide bridge was shown to induce the two oligonucleotides to bind to complementary single-stranded DNA or RNA targets with substantially enhanced thermal stability.

Clearly, coupling cationic peptides to an oligonucleotide shows promise as a means of enhancing binding affinity for target
nucleic acids. We thus proposed to prepare a small library of principally cationic peptides for conjugation to an oligonucleotide and subsequent thorough investigation of the hybridization properties of the resulting conjugates. This should allow us to directly compare the effectiveness of different amino acids in contributing to the binding of a peptide–oligonucleotide to a target strand. We have previously reported the synthesis of conjugates between an oligonucleotide and a range of biomolecules, employing a convenient methodology which involves initial preparation of cysteinyl derivatives of the moieties on a solid phase, followed by cleavage and efficient solution phase coupling to a derivatized oligonucleotide (26). Thioether and disulphide linked constructs were prepared cleanly and in high yield using this approach. In the present paper, we report on the synthesis of a series of 49 peptide–oligonucleotides based on this strategy. We describe the characterization of these conjugates and examination of their hybridization properties employing melting temperature analysis.

**MATERIALS AND METHODS**

Novasyn® TGT resin, amino acids and all reagents for peptide synthesis were obtained from Novabiochem. NAP 5 and NAP 10 Sephadex G25 gel filtration columns were from Pharmacia. SMCC was purchased from Aldrich. All buffers and solvents were of the highest available grade. All water used was filtered on a Millipore Milli-Q plus system to 18.2 MΩ cm purity. Electrospray mass spectrometry (ESMS) was carried out on a VG-Bio Q spectrometer and all data were collected and analysed using Masslynx software. Peptides were analysed in positive mode as 20 µM solutions in 1:1 acetonitrile/water. All oligonucleotides and peptide–oligonucleotide conjugates were run in negative mode as 30 µM solutions in 1:1 isopropanol/water + 1% triethylamine. Concentration in vacuo of solutions of oligonucleotides and peptides was performed using a GeneVac SF50 centrifugal evaporator with CVP100 pump. Unmodified and 5′-amino-modified oligonucleotides were purchased from Oswel DNA Service (Southampton, UK).

**Reverse phase HPLC analysis**

Reverse phase HPLC was carried out using a TosoHaas TSKgel Oligo RP 5 µm column (4.6 × 150 mm) on a Hewlett Packard series 1100 system. One gradient only was used for all samples and all runs were monitored at 254 nm. Mobile phase A was 0.1 M ammonium acetate (pH 7.0) and mobile phase B was HPLC grade acetonitrile. The gradient was isocratic 5% B for 5 min, 5–30% B in 25 min, isocratic 30% B for 5 min, 30–100% B for 5 min, isocratic 100% B for 3 min and 100–5% B for 2 min. The flow rate was 1 ml/min.

**Peptide synthesis**

Peptide synthesis was carried out in a parallel fashion in 5 ml filtration columns on a vacuum manifold (Jones Chromatography) using standard Fmoc chemistry. Each peptide was prepared on Novasyn® hydroxytrityl TGT resin (20 mg) pre-loaded with Fmoc alanine (0.19 mmol/g). Fmoc deprotection was achieved using 20% piperidine in dimethylformamide and at each coupling step 5 equiv. amino acid, 5 equiv. benzotriazole-1-yloxy-tris-pyrrrolidinophosphonium hexafluorophosphate (PyBOP®), 5 equiv. N-hydroxybenzotriazole (HOBt) in dimethylformamide and 10 equiv. diisopropylethylamine were added to the resin samples.

Each amino acid was double coupled and coupling steps were allowed to proceed for 1.5 h before washing with copious dimethylformamide, dichloromethane and diethyl ether. After each deprotection and coupling, a few beads of resin from selected syntheses were removed and subjected to the Kaiser test for free amine groups. Protected amino acid derivatives used were N-α-Fmoc-L-alanine, N-α-Fmoc-N-G2,2,5,7,8-pentamethylenechroman-6-sulphonyl-L-arginine, N-α-Fmoc-3-5-methoxytrityl-L-cysteine, N-α-Fmoc-N′-trityl-L-histidine, N-α-Fmoc-N-ε-Lysocytidine, N-α-Fmoc-δ-L-ornithine and N-α-Fmoc-N-in-t-Boc-L-tryptophan. The final Fmoc group was removed from each peptide and the resin samples were stored under desiccation at 4°C. Cleavage and concurrent deprotection of 1 mg samples of each resin were carried out in 95:2.5:2.5 trifluoroacetic acid (TFA)/trimisopropylsilane (TIS)/water (200 µl) for 3 h. Solvents were removed in vacuo and the peptides were analysed by ESMS (see Table 1).

**Synthesis of a 5′-maleimide-modified oligodeoxyribonucleotide**

The preparation of the 5′-maleimide derivatized 8mer (2) was repeated several times as and when the need for more of the oligonucleotide arose. A typical synthesis is described here.

To a solution of 198 µM 5′-amino 8mer (1) (1.01 ml, 200 nmol) in water was added 0.1 M potassium phosphate (pH 7.8) (1 ml) followed by a solution of 4-(maleimidomethyl)-1-cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC) (15 equiv., 1 mg, 3 µmol) in acetonitrile (1 ml). After 3 h at room temperature, another aliquot of SMCC was added (1 mg) in acetonitrile (100 µl) and the reaction was left to proceed overnight. The mixture was then concentrated in vacuo to <1 ml volume and made up to 1 ml with water. The solution was then applied to a NAP 10 Sephadex G25 gel filtration column pre-equilibrated in water and the oligonucleotide was eluted under gravity with 2 ml water. The product was concentrated in vacuo, made up to 200 µl with water and purified by reverse phase HPLC in two 100 µl injections. The product peak (~80% yield by comparing peak area with remaining 5′-amino 8mer peak area) was collected, concentrated to 1 ml, desalted on a NAP 10 column, quantitated by absorbance at 260 nm (A260) and evaporated to dryness to give pure 2 (120 nmol, 60% isolated yield), which was stored dry at −20°C. Re-injection of 1 nmol of the collected product onto a reverse phase HPLC column confirmed the product’s purity.

Analytical reverse phase HPLC tR 17.5 min; m/z (negative mode ESMS) mass found 2845.5 (M-H)–, calculated 2844.4.

**Preparation of peptide–oligonucleotide conjugates**

Many separate conjugation reactions were carried out during the course of this study. A typical coupling reaction was carried out as follows.

A sample of resin bearing the desired peptide (1 mg, ~200 nmol) was taken and placed in an Eppendorf tube. The peptide was cleaved and deprotected with 95:2.5:2.5 TFA/TIS/water (200 µl) for 3 h. Following thorough drying of the resin samples in vacuo for 1 h, the peptide was redissolved in 200 µl water, or 1:1 water/acetonitrile for peptides containing tryptophan, and briefly extracted with ether to remove hydrophobic impurities. An aliquot of 20 µl of the peptide solution (~20 nmol, 5–7 equiv.) was then added to a solution of the 5′-maleimide derivatized 8mer (2) (3 or 4 nmol) in 0.1 M potassium phosphate (pH 7.0) (480 µl) in 1:1 acetonitrile/water. The mixture was then concentrated in vacuo to ϼ ml volume and made up to 1 ml with water. The solution was then applied to a NAP 10 Sephadex G25 gel filtration column pre-equilibrated in water and the oligonucleotide was eluted under gravity with 2 ml water. The product was concentrated in vacuo, made up to 200 µl with water and purified by reverse phase HPLC in two 100 µl injections. The product peak (~80% yield by comparing peak area with remaining 5′-amino 8mer peak area) was collected, concentrated to 1 ml, desalted on a NAP 10 column, quantitated by absorbance at 260 nm (A260) and evaporated to dryness to give pure 2 (120 nmol, 60% isolated yield), which was stored dry at −20°C. Re-injection of 1 nmol of the collected product onto a reverse phase HPLC column confirmed the product’s purity.

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Table 1. Characterization of the peptide library and corresponding oligonucleotide conjugates by mass spectrometry, reverse phase HPLC and melt temperature analysis

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<th>Observed mass (Da)</th>
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<th>(T_m) (°C) with 16mer target(^c)</th>
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\(\text{a siliconized Eppendorf tube. The reaction was allowed to proceed for 3 h and then excess peptide was removed by passing through a NAP 5 gel filtration column, eluting with water (1.1 ml). The amount of product eluted was quantified by}\ A_{260} \text{ before analysis of 0.5 nmol by reverse phase HPLC and evaporation in vacuo. HPLC analysis typically indicated quantitative coupling had occurred. See Table 1 for isolated yields and HPLC retention times. The dried peptide–oligonucleotides were stored at }-20°C \text{ until required for melting studies. For further analysis of the conjugates 5'-AKKKK–8mer, 5'-AOOOOOC–8mer, 5'-AHH-HHHC–8mer and 5'-ARRRRRC–8mer these peptide–oligonucleotide syntheses were repeated on up to a 20 nmol scale simply by scaling up the above procedure.}

Melting temperature (\(T_m\)) studies

Melting studies were carried out in stoppered 1 cm path length quartz cells on a computer-interfaced Varian Cary 1E UV-visible spectrophotometer at 260 nm. Samples for initial analysis consisted of a 2 \(\mu\)M concentration of each oligomer in 1 ml buffer composed of 10 mM sodium phosphate (\(pH \text{ 7.0) , 0.1 M sodium chloride and 0.1 mM EDTA. Subsequent analyses were carried out in a range of buffers, namely 10 mM sodium phosphate (\(pH \text{ 6.0, 7.0, 8.0 or 10.0) , 0.1, 0.5 or 1.0 M sodium chloride and 0.1 mM EDTA. All samples were annealed by heating at }90°C \text{ for 5 min before cooling to }5°C \text{ by placing in a refrigerator for 1 h. The melting curves were measured using a temperature gradient}}\))
from 5 to 90°C with a ramp rate of 1°C/min. The cuvettes were kept under dry nitrogen to prevent water condensation at low temperature. Analysis was carried out using the Varian Thermal software and $T_m$ values were calculated using the first derivative method. Uncertainty in the $T_m$ data is estimated at ±0.5°C based on repetitions of experiments.

**Thermodynamic analysis**

The UV melting curves obtained for several oligonucleotides were analysed using the Varian Thermal software to obtain thermodynamic data. Thermodynamic parameters were estimated by fitting of the denaturation data to a two-state transition model with linearly sloping upper and lower baselines (27). The experimental absorbance versus temperature curves were converted into plots of the fraction of single strands in the duplex state ($\alpha$) versus temperature ($T$). Van’t Hoff’s plots were then derived from these $\alpha$ curves. Values of $K$, the equilibrium constant, were determined at each temperature using the equation (27,28)

$$K = \alpha/[C_T(n)^n - 1(1 - \alpha)^n]$$

(1)

where $C_T$ is the total strand concentration and $n$ is the molecularity of the reaction. Only points with 0.15 < $\alpha$ < 0.85 were included in the van’t Hoff’s plots, since this is the region where the $K$ values are most precise. For a two-state transition, if $\Delta H_{\text{DH}}$ is independent of temperature, then a van’t Hoff’s plot of $\ln K$ versus $1/T$ is found to be linear, with $(-\Delta H_{\text{DH}}/R)$ as the slope and $(\Delta S_{\text{DH}}/R)$ as the intercept. From this data, free energy values can be obtained via the relation

$$\Delta G_{\text{DH}} = \Delta H_{\text{DH}} - T\Delta S_{\text{DH}}$$

(2)

Melting curves for each of the selected conjugates were determined over at least two independent runs, the thermodynamic fitting calculations were carried out at least three times and the resulting thermodynamic values were averaged. Based on this repetition, the precision in individual $\Delta G_{\text{DH}}$, $\Delta H_{\text{DH}}$ and $\Delta S_{\text{DH}}$ measurements was estimated to be ±5–10%, which is similar to that observed in previous studies employing this methodology (29,30).

**RESULTS**

**Synthesis of a small library of cationic peptides**

The series of 49 peptides was prepared by parallel synthesis on 20 mg samples of TentaGel resin loaded with Fmoc alanine by standard Fmoc chemistry using PyBOP/HOBt as coupling agent. The peptides were all seven residues in length with the C- and N-terminal amino acids fixed and the central five positions being varied. The sequences of all the peptides prepared are shown in Table 1. Several amino acids were chosen for incorporation into the library. Lysine, ornithine, histidine and arginine were chosen as cationic amino acids due to their basic side chains, which could become protonated and enhance the affinity of the peptide–oligonucleotides for a target strand through electrostatic interaction with the phosphodiester backbone. Alanine was chosen as a neutral spacer amino acid and tryptophan was chosen as a hydrophobic amino acid which may have the potential to intercalate double-stranded DNA, as has been suggested with reference to the binding sites of certain transcription factors (31).

It also appears that at least some of the tryptophan residues of the *Escherichia coli* single-stranded binding protein interact directly with the polynucleotide and may thus contribute to the stability of the complexes (32). A monomethoxytrityl protected cysteine was incorporated at the N-terminus of each peptide to facilitate coupling to a 5’-maleimide-modified 8mer oligonucleotide after deprotection.

The library could be divided into four ‘families’; the lysine, ornithine, histidine and arginine families. Within each family of peptides the central five positions consist of two to five residues of the representative cationic amino acid interspersed with alanine or hydrophobic tryptophan residues. This permitted investigation of the effects of varying the number and positioning of the cationic amino acids and the overall hydrophobicity of the peptide on hybridization of the resulting peptide–oligonucleotide with a complementary strand. A peptide with all five variable positions occupied by tryptophan residues was also prepared to investigate the properties of an entirely hydrophobic peptide.

Synthesis of the library proceeded smoothly, as indicated by the Kaiser test for free amines (33) after all deprotection and coupling steps. Aliquots of 1 mg of each peptide-loaded resin were cleaved and deprotected with 95% TFA for characterization by ESMS. Every peptide in the library was found to have an observed mass in good agreement with the calculated value (see Table 1).

**Preparation of peptide–oligonucleotides**

The synthesis of a peptide–oligonucleotide conjugate is depicted in Scheme 1.

The oligodeoxyribonucleotide chosen for conjugation was an 8mer of sequence 5’-AATGTAAT-3’. A 5’-hexylamino derivative of this sequence (1) was converted to the 5’-maleimide derivative (2) by coupling to the bifunctional linker SMCC. Purification of

![Scheme 1](image-url)
this modified oligonucleotide by reverse phase HPLC gave the pure product in 60% yield. Coupling of this modified oligonucleotide by reverse phase HPLC gave the filtration matrix and was very difficult to elute. Thus, this

5′-amino-(1) and 5′-maleimide-8mers (2) had similar Tm values to this, indicating that attachment of the SMCC linker has no observable effect on the target binding affinity of the 8mer.

All of the peptide–oligonucleotides were observed to have enhanced binding affinity for the 16mer, the exception being the conjugate was purified directly by reverse phase HPLC. Retention times for each of the peptide–oligonucleotides prepared are shown in Table 1. As expected, increasing the number of tryptophan residues in the peptide portion led to later retention times, indicating increasing hydrophobicity. In the case of conjugates with peptides consisting of cationic amino acids and alanine alone, as the number of cationic residues increased so did the retention time. This may be indicative of interaction of the protonated side chains of the cationic amino acids with the phosphate groups of the oligonucleotide resulting in a conjugate with a lower overall (negative) charge.

A representative selection of peptide–oligonucleotides were analysed by ESMS to confirm their identity. The pentalysine conjugate and a tryptophan-containing conjugate from the lysine family were also chosen. These conjugates were collected off reverse phase HPLC, dried in vacuo to leave the ammonium salt and redissolved in 1:1 isopropanol/water + 1% triethylamine. This sample preparation procedure was found to be very effective at minimizing formation of oligonucleotide sodium adducts which are often observed in ESMS (34). Each conjugate analysed gave a mass in good agreement with calculated values. One of the ESMS spectra obtained is shown in Figure 2.

Figure 2. Transformed electrospray mass spectrometry spectrum of 5′-AAAH-HAC–8mer collected off reverse phase HPLC. Observed mass 3525.8; expected mass 3524.2.

Melting temperature analysis of hybridization with a 16mer target

The 8mer sequence used in formation of the peptide–oligonucleotide library was complementary to a 16mer of sequence 5′-ATCACATTACCATCAG-3′. This target oligonucleotide provided a 5′-single-stranded region with which the peptide portions of the conjugates may interact on duplex formation. Initial melting temperature analysis was carried out in 10 mM sodium phosphate (pH 7.0), 0.1 M sodium chloride and 0.1 mM EDTA. The Tm results under these conditions for all the peptide–oligonucleotides prepared are shown in Table 1. The Tm of the parent unmodified 8mer was found to be 19.5°C. The 5′-amino- (1) and 5′-maleimide-8mers (2) had similar Tm values to this, indicating that attachment of the SMCC linker has no observable effect on the target binding affinity of the 8mer.
lysine family), an average increase in ∼2.3 residue was estimated. Using this approach, an enhancement of arginine family to illustrate the effect of increasing the number of melting curves obtained for peptide–oligonucleotides within the alanine or tryptophan residues. Figure 3 shows a selection of arginine units were all placed together or separated by spacer peptides containing 2, 3 or 4 lysine, ornithine, histidine or any trend in affinity enhancement compared with lysine, and histidine was increase in thermal stability. Ornithine gave marginally superior family it was found that arginine residues provided the greatest tryptophan. On examination of the results from each peptide residues in the variable section of the peptide were alanine or appeared to depend solely on the number of cationic amino acid within each peptide family the calculations were based on the primary (unnormalized) data.

5′-AWWWWWC–8mer, which was found to have a $T_m$ of 19.6°C, virtually identical to the unmodified 8mer. Thus, it appears that the presence of tryptophan residues in the peptide portion of the conjugate neither enhances nor reduces target binding affinity. This observation is supported by the fact that within each peptide family the $T_m$ value of a particular conjugate appeared to depend solely on the number of cationic amino acid residues in the sequence and not on whether the remaining residues in the variable section of the peptide were alanine or tryptophan. On examination of the results from each peptide family it was found that arginine residues provided the greatest increase in thermal stability. Ornithine gave marginally superior affinity enhancement compared with lysine, and histidine was considerably less effective. The results obtained did not indicate any trend in $T_m$ based on whether the cationic residues within the peptides containing 2, 3 or 4 lysine, ornithine, histidine or arginine units were all placed together or separated by spacer alanine or tryptophan residues. Figure 3 shows a selection of melting curves obtained for peptide–oligonucleotides within the arginine family to illustrate the effect of increasing the number of cationic residues within the peptide segment.

Based on the $T_m$ values found for the pentacationic conjugate from each peptide family (e.g. 5′-AKKKKKC–8mer from the lysine family), an average increase in $T_m$ per cationic amino acid residue was estimated. Using this approach, an enhancement of ~2.3°C was observed per arginine residue. An increase of ~2.0°C per ornithine residue, 2.0°C per lysine and 0.7°C per histidine was also found. The value for arginine was in good agreement with the value of 2.0°C per residue reported by Wei et al. for a 9mer coupled to three or seven arginines (23). Poly(6-ornithine) peptides of three, eight and 12 residues linked to a 12mer oligonucleotide were reported to give an average increase of 0.5°C per ornithine residue (23). Thus, it may be that α-linked ornithine residues are better able to stabilize binding of a peptide–oligonucleotide to a target strand. However, this 12mer study was carried out using a 12mer complementary strand without a single-stranded overhang, so direct comparison is difficult.

As a control, a $T_m$ experiment was carried out on the unmodified 8mer and target 16mer with a 5-fold excess of the free peptide C-ACKKKKC-N in solution. No significant improvement in thermal stability of the 8mer–16mer duplex was observed, indicating that conjugation of the peptide directly to the 8mer was essential for enhancement of binding affinity.

**Melting temperature analysis of hybridization with an 8mer target**

The hybridization properties of the prepared peptide–oligonucleotides were further investigated by exploring the binding affinity of a selection of conjugates for an 8mer target of sequence 5′-ATCACATT-3′. The same buffer conditions of 10 mM sodium phosphate (pH 7.0), 0.1 M sodium chloride and 0.1 mM EDTA were also used in this study. Two possible binding motifs can be envisaged for a cationic peptide–oligonucleotide conjugate bound to a single-stranded target nucleic acid sequence, such as the 16mer employed earlier; one where the peptide segment interacts with the single-stranded region and an alternative arrangement where the peptide folds back and favours interaction with the duplex region (Fig. 4). This study was designed to examine the ability of the peptide to stabilize duplex formation without the presence of a single-stranded overhang region and thus, by comparison with the results obtained with the 16mer target, shed some light on which binding motif the peptide segment may favour.

The pentacationic peptide–oligonucleotides, the conjugates from each peptide family with the highest binding affinities as identified by the 16mer study, were chosen for this further analysis. The $T_m$ results obtained are given in Table 2. The parent, unmodified 8mer and oligonucleotide I were both found to have $T_m$ values of 20.0°C, which correlate well with the values observed for the 16mer target. It is clear from the results obtained for the peptide–oligonucleotides that the peptide segments are indeed able to stabilize the 8mer–8mer duplex, but to a lesser extent than was found for the 8mer–16mer duplex. For instance, the 5′-ARRRRRC–8mer was found to have a $T_m$ of 24.3°C when targeted to the 8mer, as opposed to 31.3°C when hybridized to the
The buffer used was 10 mM sodium phosphate (pH 7.0), 0.1 mM EDTA containing the relevant concentration of NaCl. This is likely to be due to partial deprotonation of N-1 of guanine and N-3 of thymine residues (which have pKₐ values of ~10.0) within the duplex strands, which would lead to disruption of Watson–Crick hydrogen bonding.

In the case of the pentahistidine peptide–oligonucleotide, the Tᵢₘ value at 1.0 M salt was 5.4°C higher than the value found at 0.1 M salt, but this conjugate still had marginally the lowest stability at high salt of the four peptide–oligonucleotides examined.

The effect of pH on hybridization to the 16mer target

The effect of varying the pH on the stability of the duplexes formed between the pentacationic peptide–oligonucleotides and the 16mer target was assessed by carrying out melting studies in buffer containing 10 mM sodium phosphate, 0.1 M sodium chloride and 0.1 mM EDTA at pH 6.0, 7.0, 8.0 and 10.0. The data obtained are shown in Table 4.

Over the pH range 6.0–8.0 the Tᵢₘ value for the unmodified 8mer remained unchanged before dropping to 15.5°C at pH 10.0. This is likely to be due to partial deprotonation of N-1 of guanine and N-3 of thymine residues (which have pKₐ values of ~10.0) within the duplex strands, which would lead to disruption of Watson–Crick hydrogen bonding.

In the case of the pentahistidine, pentaornithine and pentaarginine conjugates the melting temperatures remain largely unchanged as the pH increases from 7.0 to 8.0. At pH 10.0 there was a slight reduction in overall duplex stability, but the stabilizing effect of the cationic peptides was still considerable. Indeed, the contribution to the Tᵢₘ values from the peptide portion of these hybrids as compared with the unmodified 8mer was actually slightly greater at this elevated pH than observed at pH 7.0. For instance, the Tᵢₘ
advantage for the pentaaarginine 8mer relative to the unmodified sequence was +13.8 °C at pH 10.0 as compared with +11.8 °C at pH 7.0. These results correlate well with the pKᵦ values for the side chains of the relevant amino acids, which are ~10.5 for the amine groups of lysine and ornithine and 12.5 for the guanidine group of arginine. Thus, the side chains of these amino acids will be extensively protonated, even at the highest pH employed in this study. At pH 6.0 the Tₘ curves for these three constructs each slightly increased by ~1.0–1.5 °C as compared with pH 7.0.

For the pentahistidine 8mer, as the pH was increased to 8.0, the Tₘ dropped to 19.5 °C which was, within experimental error, identical to the value observed for the unmodified 8mer. This indicates that the peptide no longer contributed to the thermal stability of the duplex at this pH. The same phenomenon was also observed at pH 10.0. However, at pH 6.0 the Tₘ increased significantly to 28.5 °C. The pKᵦ of the imidazole side chain of histidine is ~6.5, depending on the microenvironment, which indicates that at neutral pH the histidine residues will be only partially protonated. This offers an explanation as to why the Tₘ of 22.9 °C for the pentahistidine conjugate at pH 7.0 was substantially lower than the values obtained for the other three pentacationic peptide–oligonucleotides. At pH 6.0 the histidine residues are more fully protonated, leading to a significantly raised Tₘ which is closer to, although still lower than, the values observed for the other constructs at this pH.

### Thermodynamic analysis of duplex formation

In order to gain some understanding of the influence that the peptide segment of a cationic peptide–oligonucleotide hybrid has on the thermodynamics of duplex formation with a target strand, the melting curves obtained for a selection of conjugates were further analysed to obtain thermodynamic parameters. Melting curves can be analysed to obtain estimated van’t Hoff transition enthalpies (ΔHₓ), entropies (ΔSₓ) and free energies (ΔGₓ). This was achieved by fitting of the denaturation data to a two-state transition model with linearly sloping upper and lower baselines (27). Once again, only the pentacationic peptide–oligonucleotides were investigated and the melting curves attained in 10 mM sodium phosphate (pH 7.0), 0.1 M sodium chloride and 0.1 mM EDTA were employed in the analysis. The results are detailed in Table 5.

**Table 5. Thermodynamic parameters for duplex formation of selected oligonucleotides with the 16mer target strand**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>ΔHₓ (kJ/mol)</th>
<th>ΔSₓ (kJ/mol)</th>
<th>ΔGₓ (kJ/mol)</th>
<th>Kᵦ (per M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified 8mer</td>
<td>–208.5</td>
<td>–178.2</td>
<td>–30.3</td>
<td>2.0 × 10⁵</td>
</tr>
<tr>
<td>5′-AKKKKKC–8mer</td>
<td>–221.4</td>
<td>–184.1</td>
<td>–37.3</td>
<td>3.5 × 10⁶</td>
</tr>
<tr>
<td>5′-AOOOOOOC–8mer</td>
<td>–225.2</td>
<td>–187.5</td>
<td>–37.7</td>
<td>4.1 × 10⁶</td>
</tr>
<tr>
<td>5′-AHHHHHHC–8mer</td>
<td>–213.6</td>
<td>–180.9</td>
<td>–32.7</td>
<td>5.4 × 10⁶</td>
</tr>
<tr>
<td>5′-ARRRRRRC–8mer</td>
<td>–234.0</td>
<td>–194.9</td>
<td>–39.1</td>
<td>7.1 × 10⁶</td>
</tr>
</tbody>
</table>

*The parameters were calculated from van’t Hoff’s plots derived from the melting curves obtained for each of the oligonucleotides with the 16mer target strand in 10 mM sodium phosphate (pH 7.0), 0.1 M NaCl, 0.1 mM EDTA buffer.*

In the case of the unmodified 8mer the obtained ΔHₓ value of –208.5 kJ/mol was found to be in good agreement with a theoretical value of –209.2 kJ/mol calculated using nearest neighbour thermodynamic values (37), indicating the validity of the van’t Hoff’ s plot thermodynamic analysis we employed. As expected from the thermal stability data, all four pentacationic peptide–oligonucleotides exhibited a more favourable free energy change on binding as compared with the unmodified 8mer. This free energy advantage (ΔΔGₓ) ranged from –2.4 to –8.8 kJ/mol (at 298 K) for the pentahistidine and pentaaarginine conjugates respectively, corresponding to an ~2.5- to 35-fold enhancement in association constant. The increased free energy of binding observed for each conjugate was found to arise from a more favourable enthalpic term. The order of enhancement in ΔHₓ for the pentaaarginine conjugate > pentanolysine ≥ pentalysine > pentahistidine, which was in good agreement with the order observed for the Tₘ values. The actual enhancement values found (ΔΔHₓ) were ~25.5, ~16.7, ~12.9 and ~5.1 kJ/mol respectively.

To ascertain whether increasing the degree of protonation of the pentahistidine conjugate would result in an improved free energy and more exothermic enthalpic contribution, thermodynamic analysis was carried out on the melting curves obtained at pH 6.0. This indeed indicated an enhanced ΔGₓ (298 K) value of ~36.8 compared with ~32.7 kJ/mol at pH 7.0, representing an increase in the association constant (Kᵦ) from 5.4 × 10⁵ M (pH 7.0) to 2.8 × 10⁶ M (pH 6.0). The origin of this improvement was found to be a more exothermic ΔHₓ contribution of ~222.5 kJ/mol. The corresponding ΔHₓ values for the unmodified 8mer, pentalysine, pentanolysine and pentaaarginine conjugates at pH 6.0 were found to be ~210.0, ~227.0, ~228.4 and ~238.4 kJ/mol respectively.

### DISCUSSION

Two different strategies are generally employed in the synthesis of peptide–oligonucleotide hybrids. In the total synthesis approach, the peptide and oligonucleotide portions are synthesized sequentially on the same solid support, connected by some form of linker (11,38). An alternative strategy is to prepare the peptide and oligonucleotide segments separately and couple them together post-synthetically, usually connected by a bifunctional linker (12,39). We have used a post-synthetic approach which has the advantage of enabling coupling of a range of different peptides to a single oligonucleotide sequence, principally due to the excellent efficiency of the coupling reactions and the ease of purification. Studies of hybridization of the library of 8mers with a 16mer target at pH 7.0 and 0.1 M sodium chloride indicated that all four of the cationic amino acids investigated enhanced binding affinity. The order of stabilization was found to be arginine ≥ ornithine > lysine > histidine. It can be postulated that this stabilization is predominantly due to electrostatic interaction between the cationic amino acid side chains and the DNA. This conclusion is supported by the observations made in the pH studies that suggest that the enhancement of duplex stability is directly related to the protonation state of the amino acid side chains. The stabilities of most protein–nucleic acid complexes, both sequence-specific and non-specific, are known to have large contributions from electrostatic interactions (40). Our observation that tryptophan was found to neither stabilize nor destabilize duplex formation in our studies would appear to be in agreement with the studies of Mascotti and Lohman, who observed that...
tryptophan interactions resulted in no significant effect on $\Delta G^\circ_{\text{obs}}$ due to entropy–enthalpy compensations (41). Altering the position of the cationic amino acids within the variable region of the peptide segments appeared to have no effect on binding affinity. Thus, the thermal stability of the constructs simply depended on the number and identity of the cationic amino acids within the peptide and not on their sequential arrangement.

Hybridization of the pentacationic peptide–oligonucleotide conjugates to an 8mer target strand indicated that the peptide portion was still able to stabilize duplex formation in the absence of a single-stranded region, but the degree of stabilization was reduced. Indeed, less than half of the stabilization in terms of °C per cationic residue was obtained for each of the four different amino acids as compared with the values obtained in the 16mer binding experiments. This is in agreement with the observation that oligoarginine and oligolysine peptides have been shown to associate more strongly with single-stranded polynucleotides than supercoiled or linear duplex DNA (41,42). It therefore seems likely that in the case of duplex formation with the 16mer, it is energetically more favourable for the peptide portion of the conjugates to be associated with the single-stranded overhang rather than binding to the double-stranded region, imparting greater overall stability to the complex.

The salt dependence of hybridization to the 16mer target at pH 7.0 for the selected polycationic conjugates was also evaluated. Increasing the concentration of sodium chloride from 0.1 to 1.0 M resulted in a significant rise in the $T_m$ value for the unmodified 8mer, consistent with observations made for other DNA duplexes (35,36). This is in agreement with polyelectrolyte theories, such as the counterion condensation theory (36,43,44), that describe duplex DNA as having a higher charge density as compared with the single-stranded form. Hence, helix formation is accompanied by an increase in counterion association. An increase in bulk salt concentration stabilizes the state with the higher charge density as compared with the single-stranded state, resulting in an increase in $T_m$ for the helix-to-coil transition. In the case of the four pentacationic peptide–oligonucleotides, increasing the salt concentration resulted in a decrease in the contribution of the peptide portion to the overall $T_m$, as determined by comparison to the values obtained for the unmodified 8mer. These results can be related to the findings of Mascotti and Lohman (41,42), who noted that for oligolysine and oligoarginine peptides, increasing concentrations of salt resulted in decreasing association with polynucleotides. Our observations are also consistent with the proposal that the increased binding of the peptides to the nucleic acid at lower salt concentrations is driven primarily by cation release from the nucleic acid into bulk solution, which results in an increased entropy of dilution (44,45). Thus, for the polycationic peptide–oligonucleotides there may be two countervailing influences on the $T_m$ of duplex formation as the concentration of salt increases, namely increasing stability of the double-stranded DNA region and decreasing association of the peptide portion with the oligonucleotide target.

The pH dependence of the $T_m$ values for the pentacationic conjugates binding to the 16mer target was related to the pH of the ionizable side chains of the amino acids. It is interesting to note that for the pentahistidine construct the $T_m$ increased markedly (+9.0°C) on decreasing the pH from 8.0 to 6.0, in line with the $pK_a$ of the imidazole side chain (~6.5). There may be potential to exploit this property as some form of sequence-specific ‘pH hybridization switch’ that is very sensitive to pH changes around pH 7.

Thermodynamic analysis of hybridization of the selected pentacationic peptide–oligonucleotide hybrids indicated that each exhibits a more negative free energy change on target binding as compared with the unmodified 8mer due to a more favourable enthalpic term. The least favourable $\Delta H_{\text{HH}}$ value was obtained for the polyhistidine conjugate. However, on decreasing the pH from 7.0 to 6.0 the $\Delta G_{\text{HH}}$ (298 K) parameter improved from ~32.7 to ~36.8 kJ/mol and the $\Delta H_{\text{HH}}$ contribution changed from ~213.6 to ~222.5 kJ/mol. This suggests that a major contribution to the binding free energy for these peptide–oligonucleotides is due to the formal positive charge on the amino acid side chains. This hypothesis is further supported by the observation that the uncharged pentatryptophan conjugate (Table 1) and the pentaarginine conjugate at pH 8.0 and above display no enhancement in $T_m$ over the unmodified 8mer (Table 4). However, it is unlikely that positive charge alone is responsible for the enhanced binding energy, since the $T_m$ observed for the pentaarginine conjugate was significantly higher than the values obtained for the pentalysine and pentaornithine peptide–oligonucleotides, even at low pH, where all the cationic residues would be fully protonated (Table 4). This is further illustrated by the free energy values obtained at pH 7.0, which indicate that the pentaarginine conjugate has a more favourable $\Delta G_{\text{HH}}$ (298 K) value than those obtained for the pentalysine and pentaornithine examples, which are not significantly different. The free energy advantage is gained from a more favourable enthalpic term, with the enthalpic advantage ($\Delta H_{\text{HH}}$) over the unmodified 8mer being ~26.5, ~16.7 and ~12.9 kJ/mol for the pentaarginine, pentaornithine and pentalysine conjugates respectively. Consistent with our observations, previous studies have revealed that oligoarginines bind systematically with a higher affinity to polynucleotides than oligolysines possessing the same net charge and that this higher affinity was enthalpic in origin (41). These effects were independent of base composition and polynucleotide type and it was concluded that the extra binding energy was derived from more enthalpically favourable interactions of arginine with the phosphate backbone. It was proposed that the most likely major contribution to this increased stability would be hydrogen bonding between the guanidinium group of arginine and the nucleic acid phosphate groups. However, it has been shown that for the dipeptide Arg-Glu, the arginine residue can form hydrogen bonds with both cytosine and guanosine residues in single-stranded nucleic acids and guanosines in duplex nucleic acids, as well as with nucleic acid phosphates (46). The interactions between the guanidinium side chain of arginine (and related structures) and nucleic acids has been exploited in a number of nucleic acid binding systems. The minor groove binding agent netropsin possesses terminal guanidinium and propylamidinium groups which contribute to binding of the molecule with double-stranded DNA. The crystal structure of netropsin bound to a double helical B-DNA dodecamer indicated that the binding of these two groups involved hydrogen bonding with N-3 of adenine residues as well as an electrostatic component (47). Also, in addition to carrying a positive charge, the arginine guanidinium group is capable of forming bifurcated hydrogen bonds (48) which can potentially enhance the stability of protein–nucleic acid complexes (49). Binding specificity derived from this hydrogen bonding capability has also been demonstrated for arginine-containing peptides. Of particular note
is a peptide fragment of the HIV-1 Tat protein, which binds with sequence specificity to its mRNA recognition site, the TAR element (50–52). This specificity is dependent on the presence of an arginine residue at a specific position within the nonomer peptide. The favourable interactions between guanidinium groups and the phosphodiester backbone of nucleic acids have been exploited by Dempcy et al. in the design of deoxynucleic guanidine (DNG) oligonucleotides (53). This DNA analogue possesses 5′-3′ guanidyl linkages in place of the natural phosphodiester bridges and has been shown to bind target DNA sequences with extraordinarily high affinity. Molecular modelling studies of a decamethylene DNG strand binding to a complementary DNA sequence suggested that the backbones of the two strands approach one another due to electrostatic interactions between repeating phosphate and guanidinium groups.

Further investigations will be carried out on the DNA–peptide conjugates to study the kinetics of binding and the specificity of polynucleotide recognition. We have recently demonstrated quantitative coupling of a peptide from a single bead with oligonucleotide 2 which should make it possible to generate larger libraries of peptide–oligonucleotides using a combinatorial split synthesis approach.

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See supplementary material available in NAR Online.

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